Soluble Expression of Bladder Cancer Biomarker Matrix Metalloproteinase 1

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Background: Matrix metalloproteinase 1 (MMP1) has been shown as a novel unique biomarker of bladder cancer in urine. MMP1 can only be detected using conventional and time-consuming methods, such as ELISA and Western. Refolded MMP1 has been achieved and used in probe screen for many years, while there is no clinical application for MMP1 detection until now. Soluble expression of MMP1 is necessary in urine detection. **Methods:** cDNA of MMP1 has been isolated from human embryonic kidney 293 (HEK293) cells. The catalytic domain of MMP1 is expressed as fusion protein with *Escherichia coli* thioredoxin (TrxA). The 30 kDa recombinant proteins were pu-

rified by Ni-chelating chromatography. The activity of soluble MMP1 was determined and compared with refolded MMP1 by zymography. **Results:** Compared with refolded MMP1, TrxA can increase the solubility of MMP1. The soluble MMP1 has the same protein sequences with refolded MMP1 and increased 1.54-fold of gelatin-degradation activities than refolded MMP1. **Conclusion:** Successfully soluble expression of MMP1 has been achieved by fusion expression and will make progress in discovering specific molecular probes against MMP1. J. Clin. Lab. Anal. 29:275–280, 2015. © 2014 Wiley Periodicals, Inc.

Key words: bladder cancer; biomarker; prokaryotic expression; MMP1

INTRODUCTION

In China, increasing numbers of males' death cases reported are due to bladder cancer. Despite recent improvements in diagnostic and therapeutic techniques, the survival rate of bladder cancer patients remains poor due to posttreatment recurrence of disease (1, 2). The lack of effective detection techniques for recurrence of prostate cancer is related to a large extent to a poor understanding of the molecular changes underlying the progression of the disease toward invasion and metastasis (3, 4). Thus, the development of new technique for detection of new biomarkers will improve clinical management of bladder cancer.

Matrix metalloproteases (MMPs) are a family of calcium- and zinc-dependent proteases that play pivotal role in regulation of the cell microenvironment and cell behavior. The high-level expression of the active MMPs are generally associated with tumor process, including growth, migration, invasion, and metastasis (5–7). MMP1, also known as interstitial and fibroblast collagenase, plays important roles in physiological and patho-

physiological processes, such as embryonic development, tissue remodeling, arthritis, and metastasis, through degrading of the extra cellular matrix, including interstitial collagens, types I, II, and III (8). Elevated MMP1 expression has been identified to be associated with pancreatic, prostatic, colorectal, and breast cancer (9–12). Recently, MMP1 in urine samples of bladder cancer patients has been identified as a new biomarker for predicting which patients may develop advanced stage or grade bladder cancer (6, 13–15). Although the detection of MMP1 in urine samples can be completed using ELISA and concentrated urine sample, the manipulation of sample

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preparation and detection are complex and time-wasting. Easily manipulated detection technique using small MMP1 probe is needed urgently.

In order to express properly folded MMP1 for functional probe screen, recombinant expression has been applied to obtain active MMP1. Many researchers have expressed the recombinant MMP1 with high activity through refolding of inclusion bodies in *Escherichia coli* (*E. coli*; (16–18)). Soluble expression of MMP1 has been reported by Lu et al. recently (19). Soluble expressed MMP1 is most close to its native structure. Despite the availability of numerous soluble expression systems, recombinant protein expression remains difficult. Establishing the best soluble expression method for different proteins remains empirical. Expression of proteins fused with *E. coli* thioredoxin (TrxA) has been reported to increase the solubility and activity of various proteins, such as insulin, antibody, and antimicrobial peptide. Herein, we report a new method for soluble expression of catalytic domain of human MMP1 in *E. coli* BL21 (λ DE3). Our results showed that TrxA can also increase the solubility and activity of MMP1. The purified soluble MMP1 has much more activity in digesting substrates than refolded MMP1.

MATERIALS AND METHODS

Clone of MMP1 cDNA and Construction the Expression Plasmids

HEK293 cells were grown in DMEM (Dulbecco's essential medium with Earle's salts) supplemented with 10% fetal bovine serum (Hyclone, defined), glutamine, penicillin and streptomycin.

TrxA-contained expression vector pET-32a (Novagen, TAKARA Biotechnology (Dalian) Co., Ltd.) has been chosen for basal platform of MMP1 and TrxA fusion expression. As there are more redundant tags and sequences in pET-32a, a modified vector, pET-32a', has been constructed by reinserting the TrxA gene that was amplified by PCR (Polymerase chain reaction) using forward primer (5'-GATATACATATGAGCGATAAAATTATTTCAC-3') and reverse primer (5'-CCATGGGGCCAGAACGAGAACCGGCCAG-3') between the Nde I and Nco I sites. MMP1 gene was cloned from HEK293 cDNAs. Briefly, total RNA of HEK293 cells was isolated by Trizol reagent (TAKARA) according to the manufacturer's instruction. For RT-PCR, cDNA was synthesized from the total RNA by using an AMV RNA PCR Kit (TAKARA, Biotechnology (Dalian) Co., Ltd.). The cDNA was amplified by PCR with forward primer (5'-ATGCACAGCTTTCCTCCACTGCTG-3') and reverse primer (5'-TCAATTTTTCCTGCAGTTGAACAG-3'). The catalytic domain was amplified by PCR

using forward primer (5'-ATGCCATGGACGACGACGACAAGTTTGTCTCACTGAGGGGAAC-3') and reverse primer (5'-CCGCTCGAGACGTCCATATATGGCTTGG-3'). The amplified fragment was digested with Nco I and Xho I, then cloned into vectors pET-28a and pET-32a'. The gene was sequenced by Sangon Ltd. (Shanghai, China).

Expression of the MMP1 Protein

The expression vector was transformed into *E. coli* BL21 (λ DE3) cells. The transformed cells were cultured overnight in 3 ml LB medium containing 100 μ g/ml ampicillin, and diluted (1:50) in fresh LB (100 μ g/ml ampicillin) at the next day morning, and incubated at 37°C with shaking (180 rpm) until OD₆₀₀ was 0.6–0.8. In order to induce the expression of constructed proteins, 0.1 or 1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) was added into the culture medium. The cells were then induced at 25°C for 2 hr or 37°C for 4 hr with shaking (180 rpm). Cells were collected and washed twice by 20 mM Tris pH 8.0, the pellet was harvested by centrifugation at 6000 rpm at 4°C and frozen in –80°C overnight.

Purification of Soluble MMP1 and Refolding of MMP1 Inclusion Bodies

For soluble expression proteins, the frozen cell pellets were resuspended in 20 mM Tris pH 8.0 containing 1 mg/ml lysozyme and incubated at room temperature for 30 min with gentle shaking. The sample was then centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant and the insoluble cell debris were subsequently collected and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The soluble fraction obtained above was dialyzed overnight in 20 mM Tris-HCl pH 7.5. The TRX-MMP1 was purified by affinity chromatography using Chelating Sepharose Fast Flow (prepared according to the application note of GE Healthcare, 18-1134-37 AC). The fractions containing the highest amount of fusion protein were collected in elution buffer (50 mM pH 8.0 Tris, 0.5 M NaCl, and 100 mM imidazole) and dialysis with cleavage buffer (50 mM pH 8.0 Tris, 0.5 M NaCl). TrxA tags are removed by enterokinase cleavage. Briefly, 50 μ g purified soluble TRX-MMP1 was cleaved by one unit enterokinase at 16°C for 12 hr. The MMP1 proteins were purified (or TrxA proteins and enterokinase were removed) by pass-through a Chelating Sepharose Fast Flow column.

For inclusion body proteins, the frozen cell pellets were resuspended in 20 mM pH 8.0 Tris, and lysed by ultrasonic at 50% power for 20 min with 30 sec pulse and 30 sec interval. The sample was then centrifuged at 12,000 rpm for 10 min at 4°C. The inclusion bodies were washed three

Fig. 1. Schematic illustration of MMP1 soluble expression construction.

times with 20 mM Tris buffer (pH 7.5) containing 1% Triton X-100, 100 mM NaCl, and 1 M urea. The washed inclusion bodies were dissolved by stirring for 2 hr in 20 ml of 20 mM pH 7.5 Tris with 8 M Urea. The solubilized inclusion bodies were centrifuged at 12,000 rpm for 10 min at 4°C and purified by Ni-chelating sepharose FF chromatography. The fractions containing the highest amount of fusion protein were collected in elution buffer (50 mM pH 8.0 Tris, 0.5 M NaCl, and 100 mM imidazole) containing 8 M Urea. The purified MMP1 inclusion body was dialyzed with elution buffer without imidazole and the concentration of proteins were diluted into approximately 100 µg/ml with dilution buffer (pH7.5, 20 mM Tris, 10 mM DTT, and 8M Urea). The protein solution was then dialyzed in the dilution buffers with different urea concentrations (6M, 4M, 2M) for 2 hr per solution. Finally, the protein was refolded by dialysis in refolding buffer (pH7.5, 50 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, 20 µM ZnSO₄, and 0.05% Brij-35) for 12 hr at 4°C.

Gelatin Zymography

Gelatin zymogram was performed in 10% nonreducing SDS-PAGE with 0.15% gelatin. After electrophoresis, SDS was removed by 2.5% Triton X-100. Gel was then

washed and incubated in incubation buffer (50 mM Tris, pH 7.5, 0.01% Brij-35, 5 mM CaCl₂, and 1 µM ZnCl₂) overnight at 37°C. Gels were stained with 0.5% Coomassie Blue R-250. The relative gelatin degradation activities were semiquantified according to the method (20).

Fluorescence Substrate Degradation

DQ gelatin was prepared as a 110 mg/ml stock solution in reaction buffer containing 10 mM Tris, 100 mM NaCl, 5 mM CaCl₂, and was added to each assay well of a 96-well plate. The final volume in each well was 100 µl. The final substrate concentration was 10 µg/ml. A desired volume of MMP1 was added to appropriate wells. The reaction buffer without MMP1 was used as a negative control. The reactions were started by the addition of 1 µl of DQ-gelatin. Plates were read by using the fluorescent microplate reader at $\lambda_{ex} = 495$ nm and $\lambda_{em} = 515$.

RESULTS AND DISCUSSION

Construction of Expression Vectors

Total RNA of HEK293 cells has been prepared, and the cDNA fragment of MMP1 was amplified from HEK293 cells step by step according to the instructions of cDNA

Fig. 2. Expression and purification of soluble TrxA-MMP1 and MMP1 refolded from inclusion body. (A) The effect of inducing time on the expression level of TrxA-MMP1 and MMP1-His6. The MMP1 inclusion bodies increased along with inducing time in 4 hr, while the TrxA-MMP1 expression became saturated in 2 hr after induced by IPTG. (B) In total, 1 mg proteins of TrxA-MMP1 and MMP1 expression strains have been loaded on lanes 2 and 4 (P). The equal amount of soluble fractions were loaded on lanes 1 and 3 (S). Asterisk represents the soluble TrxA-MMP1 proteins and open circle represents the inclusion proteins MMP1-His6.

synthesis Kit (Transgen Ltd.). Sequence of MMP1 catalytic domain has less sequence similarity compared with other MMPs. Thus, the catalytic domain of MMP1, with a Mr of 550 bp, had been amplified by PCR with primers as indicated in Materials and Methods, and inserted into various expression vectors including pET-15a, pET-22b, pET-28a, and pET-32a. The result showed that MMP1 was expressed as inclusion body in pET-15a, pET-22b, and pET-28a, and soluble expressed in pET-32a, indicated TrxA could increase the solubility of MMP1 in *E. coli*. TrxA tag has been demonstrated to effectively increase the solubility of many other proteins in *E. coli*. As more undesired protein tags and sequences in the pET-32a vector, a modified vector pET-32a' has been constructed, and

Figure 1 shows the schematic diagram of soluble expression construction. Finally, the MMP1 has been expressed in soluble fractions in vector pET-32a' and as inclusion body in pET-28a for comparison.

Protein Expression

Expressions of MMP1 in *E. coli* have been reported by several laboratories in kind of inclusion body. Herein, we expressed catalytic domain of MMP1 construct employed a His-tag, MMP1-His6, in the vector pET-28a. The expression of MMP1-His6 has been investigated, and 1 mM IPTG induced high concentration of inclusion body of MMP1 in 4 hr at 37°C. And the amount of inclusion body proteins increased along with the time. By contrast, the expression of TrxA fused MMP1-His6, TRX-MMP1, which is expressed in pET-32a', gets its balance on 2 hr after inducing by 0.4 mM IPTG (Fig. 2A). The SDS-PAGE showed the MMP1-His6 was mainly expressed in kind of inclusion body in the pellet of cell lysate, and TRX-MMP1 expressed mainly in the soluble fraction of cell lysate (Fig. 2B).

Fig. 3. The purification and gelatin-degradation activity of soluble MMP1. (A) The purified proteins in Trx soluble system and inclusion body system. In total, 2.5 μ g purified fusion protein TrxA-MMP1 has been loaded on lane 1, and cleaved by EK. Lane 2 presented the MMP1 that is cleaved and purified from fusion protein TrxA-MMP1. Two micrograms refolded MMP1 has been loaded on lane 3 as a control and lane 4 presented the removed fractions from lane 1 (cleaved TRX tags and invisible EK), respectively. (B) The gelatin degradation of purified proteins. Lane 1 presented 2.5 μ g purified TrxA-MMP1. Lanes 2 and 3 presented the purified MMP1 and removed fractions from lane 1 (cleaved TRX tag and invisible EK).

Protein Purification and Activity Analysis

Due to the His tag at the C-terminal end of the both proteins MMP1-His6 and TRX-MMP1, the purification of these proteins was performed according to the manufacturer's instruction of Chelating Sepharose Fast Flow column as indicated in Materials and Methods. For TRX-MMP1, the soluble fractions of cell lysate induced for 2 hr have been collected, and purified by Ni-chelating Sepharose Fast Flow column. Figure 3A shows 30 kDa

purified TRX-MMP1 on lane 1, and the 10 kDa TrxA tag band on lane 4 that was cleaved by enterokinase and separated from TRX-MMP1. After cleavage, the soluble MMP1 (Fig. 3A lane 2) has been purified by Ni-chelating Sepharose Fast Flow column. The gelatin zymogram (Fig. 3B) showed that the purified soluble MMP1 and TRX-MMP1 proteins both have the gelatin-degradation activity. For MMP1-His6, the inclusion body has been collected and refolded as the Materials and Methods indicated. The SDS-PAGE showed that the purified refolded MMP1 gave one clear band at 20 kDa whose molecular weight is equal to the soluble MMP1. The semi-quantify gelatin zymogram showed the relative gelatin degradation activities of soluble MMP1 was 1.54-fold of refolded MMP1 (Fig. S1). Fluorescence substrate degradation has also been employed to compare the activity between soluble and refolded MMP1. The result showed the soluble MMP1 has higher substrate degrade rate than refolded MMP1 (1.8×10^5 mF.U./min compared to 1.1×10^5 mF.U./min; Fig. S2). These results indicated that the TrxA fusion expression system has more advantages than refolding from inclusion body in MMP1 expression in *E. coli*.

CONCLUSIONS

MMP1 has been identified as one of the most highly upregulated proteins in a variety of cancers, including colorectal, esophageal, pancreatic, gastric, breast, and malignant melanoma (11, 12, 21). The detection of MMP1 in urine of bladder cancer patients is very important as implied by recent researches (6, 10, 13). Screen of probes against MMP1 have been applied to detect MMP for decade years. But there is no product in urine detection. The probable reason was the refolded MMP1 used in screen has different structure with the native MMP1 in urine sample. High expression of exogenous proteins in *E. coli* usually causes the formation of inclusion bodies, and the recombinant protein structure and activity are determined enormously by the refolding process. As many proteins are difficult to be soluble expressed, various soluble expression systems have been developed at many facets. Researchers are trying to solubly express the MMP1 by increasing the soluble fraction of inclusion body (19). In this study, we found TrxA can increase the solubility of MMP1 in the cytoplasm of the bacteria. As a control, inclusion body of MMP1 has been studied at meantime. The activity of soluble MMP1 was higher than the refolded MMP1. These results indicated MMP1 can be soluble expressed in cytoplasm of *E. coli* by fusion with TrxA tag, and confirmed that TrxA tag did help heterogeneous protein folding correctly. The soluble MMP1 have more activities than the refolded one and may benefit in vitro screening of small probes against MMP1.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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